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Differentiation of *Helicobacter pylori* using PCR-RFLP

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1. Abstract

Helicobacter pylori is one of the most common human pathogens and since it was discovered in 1982 by Marshall and Warren has been established as one of the most variable and diverse bacteria known to man. Resident to the lining of the human stomach it is seen as the cause of a multitude of illnesses, ranging from mild gastritis, gastroduodenal ulcers as well as gastric cancer and controversially even heart disease. Some links have even been made to a protective function concerning people infected with *Helicobacter pylori* being less likely to suffer from GERD (gastro-esophageal reflux disease). The differentiation of a pathogen so prevalent and variable presents a challenge. To date two versions of *Helicobacter pylori* have been sequenced indicating an immense variability in its genetic makeup. Sequencing cannot however be effectively used to differentiate this bacterium in an everyday setting and is too cost- and time-intensive to describe the multitude of strains that have been found. PCR-RFLP (polymerase chain reaction used as amplification and restricted fragment length polymorphism used as differentiation method) is a tried and tested method of differentiating bacteria. We propose that from 37 *Helicobacter pylori* positive gastric biopsies and 33 viable PCR products the selection of two different genes (*UreAB* and *UreCD*) and consecutive PCR-RFLP on these two genes (using *HaeIII* and *NdeII* respectively) is superior to PCR-RFLP on only one gene. After running the experiment we include an analysis of the resultant gel electrophoresis band patterns with GelComparII to our visual evaluation. We can demonstrate that the two combined PCR-RFLP runs are superior to both of the single runs with only one pair of *Helicobacter pylori* strains showing an identical band pattern in both *UreAB/HaeIII* and *UreCD/NdeII* runs. The ideal combination of differentiation methods and computing equipment would allow the construction of a database correlating pheno- and genotype. This would allow the clinician to make a more precise judgement on which patient it is necessary to treat, therefore reducing costs to society and slowing down the emergence of resistance to antibiotics.

2. Introduction

When confronted with *Helicobacter pylori*, the initial reaction is to state that it is a Gram-negative, curved or spiral flagellated bacteria that inhabits the mucus layer of the human gastric epithelium (Marshall, 1989). This does not do *Helicobacter pylori* justice. More than just being a pathogen that has to be eliminated it offers researchers a unique opportunity to study the finely tuned interplay of the human body and an organism residing there within.
It is commonly found in 30-90% of the population depending on socio-economic conditions and in all countries tested to date (Taylor et al., 1995). Being the cause of and associated with diseases such as duodenal ulcer, chronic active gastritis (Marshall et al., 1984), gastric cancer (Nomura et al., 1991), MALT-lymphoma (Wotherspoon et al., 1993) and controversially even ischemic heart disease (Mendall et al., 1994) it represents a challenge for the human body. Considering the abundance of infection and the relatively low count of illnesses it is associated with there must be some variables that account for the discrepancy. The following text is in the context of finding those variables.

This introduction seeks to give a broad overview of the discovery of *Helicobacter pylori*, a description of the pathogen itself and to discuss its importance for the medical community, as well as highlight the goals of the research undertaken in this paper.

2.1 Discovery

Development of microscopic and consequently bacterial science brought about new areas of research. The presence of gastric spiral bacteria was first reported in 1893 by Bizzozero, an Italian physician. His research into the intestine of canines revealed the presence of spiral bacteria in the gastric mucosa, the presence of which was later supported by Solomon, who found similar bacteria in the stomachs of cats and mice. The first demonstration of these bacteria in human stomachs took place in 1906 and was done by Krienitz. He had been researching patients suffering from gastric carcinoma, in whose stomachs he commonly isolated the previously described bacteria. Nine years later spiral micro organisms in the stomachs of patients with gastric and duodenal ulcers were reported (Rosenow et al., 1915), but both he and Krienitz considered them to be contaminants to rather than the cause of those diseases. Bacteriology was still in its infancy at the time and penicillin not in common usage for many years to come and the cat of knowledge was pursued down different alleys.

In 1938 *spirochetes* were found in 43% of 240 human and other primate stomachs after autopsy using a Haematoxylin-Eosin stain (Doenges et al., 1938), without deriving any association to an illness. This heightened interest in spiral bacteria brought forth another interesting article written only a couple of years later (Freedberg, 1940), which found a population of gastric cancer victims to have been colonised by *spirochetes* in 37% of all cases. But when in 1954 an extensive study of 1000 gastric biopsy specimens revealed no correlation between gastric cancer, ulcers of both the stomach or the duodenum and *spirochetes*, interest in the bacteriology of the stomach faltered and waned.

Other paths of research were paving the way for the discovery of *Helicobacter pylori*. In 1924 urease activity in the stomach was first described by Luck et al., but they were unable to elaborate on its function. A generation later Fitzgerald et al., 1950 suggested that urease activity protected gastric mucosa by mediating a reaction between urea and hydrogen ions, thus neutralising gastric acid by producing ammonia. They even went so far as to administer urea to test subjects in order to prove the validity of their theory. The bacterial origin of gastric urease was demonstrated by Delluva in 1968, when the absence of urease could be proven by testing germ free animals as urease negative.

Both areas and the symptomatic treatment of ulcer patients found its culmination in Marshall and Warren's discovery of *Helicobacter pylori* in 1982.

For the past decades ulcer patients had been treated according to the dictum by the Croatian physician Karl Schwarz (1910) 'No Acid, No Ulcer'. New effective treatments such as stilboestrol and H_2-receptor antagonists were at hand in the sixties and seventies. But, they had to be taken for life, since upon cessation of therapy ulcers would invariably reappear.

In 1975 spiral bacteria were reported in association with gastritis in 80% of gastric resection specimens from patients with gastric ulceration (Steer et al., 1975), but culture proved unsuccessful. Four years later similar bacterial specimens were found in the luminal surface of epithelial cells of gastric ulcer patients (Fung et al., 1979): Concerned largely with the correlation of endoscopic, histological and ultrastructural findings in chronic gastritis the bacteria were noted in passing.

This was the professional environment that Professor Robin Warren, a histopathologist at the Royal Perth Hospital in Western Australia worked in at the time. In 1979 he had begun to notice that curved bacteria were often found in gastric biopsy specimens taken from patients with gastritis submitted for histological examination when slides were stained with the Warthin-Starry agent. These organisms were not present within the gastric mucosa, but lay in the mucus layer above the tissue (Marshall, 1989). Correlating these findings with previous reports encouraged further research. The problem at the time was that of unsuccessful culturing which had frustrated scientists previously working in that field, the bacteria had been left aside until generations of physicians forgot about it. Barry Marshall, a second year medical internist, became interested in Prof. Warren's findings and they began a research project in 1981 with the goal of isolating and culturing the bacteria. Since the bacteria were curved, Gram-negative, microaerobic rods they used culture protocols applied to the isolation of the *Campylobacter* species which they closely resemble. Initially without success. They were however reassured that they were on the right track when Marshall successfully treated an elderly Russian suffering from gastritis and having histologically proven infestation with spiral bacteria with tetracycline. Symptoms failed to return after cessation of therapy.

Progress on culturing the spiral bacteria continued to be unsuccessful. One Easter holiday culture plates were left in an incubator by mistake and on return almost discarded, because the

Campylobacter-specific protocols used had a three-day incubation period and they had been locked in for two days too many. To their surprise and delight they found that growth had occurred and they now had visible colonies of the bacteria they were looking for. In the following months they successfully isolated strains of bacteria from biopsy samples of 11 patients with gastric disease using the new protocol.

It was considered that they had enough material to present their findings on the relation between these *Campylobacter*-like organisms and intestinal disease to a wider audience. The 1982 conference of the Royal College of Physicians was the venue for their presentation and not surprisingly it evoked mixed feelings in the assembly. The association of the bacteria with chronic active gastritis, peptic ulceration and possibly gastric adenocarcinoma was first published in letter form in 1983, then in the seminal work 'Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration' in the Lancet in 1984, where they proposed to call them *Campylobacter pyloridis*.

The medical community at large was not easily convinced of the notion that peptic ulcers were supposedly of bacterial origin. Previously it had been considered that an imbalance of acid production in the stomach somehow related to a dysfunction of the stomach itself was the cause, and an external pathogen did not fit the picture. More work was needed to provide unequivocal proof.

In the same year, Rollason's et al., 1984, a retrospective study of 310 endoscopic gastric biopsies, revealed 42% histological correlation of gastritis and existence of *Campylobacter pyloridis*, these being confined to the surface of the glands and epithelium. The following year Marshall (Marshall et al., 1985) supplied a study on two years biopsy samples of the gastric antrum which were cultured for *C. pyloridis*, detecting it in 88% of patients with histologically confirmed gastroduodenal disease and providing negative cultures for all patients with normal mucosa. He argued that pyloric *Campylobacter* infection be a major factor in the development of dyspeptic disease and peptic ulcer. Marshall provided himself with the ultimate argument in favour of pathogenicity of *C. pyloridis* by attempting to fulfil Koch's postulates for an infectious agent. The healthy young man had biopsy-proven normal gastric tissue before the experiment, but after oral ingestion of pure cultured bacteria he developed a mild illness which lasted for 14 days; he experienced a light fever, tiredness and a loss of appetite. Histology done on a biopsy taken at the tenth day after ingestion showed gastritis but this had largely resolved by the fourteenth day. It was proposed that had *C. pyloridis* not been eradicated using antibiotics the chronic infection would have led to peptic ulceration (Marshall et al., 1985).

In another study (Goodwin et al., 1986) lesions found in histological samples of *C. pyloridis*-infected stomachs were compared to those produced by enteropathogenic *Escherichia coli* and found to be similar. Goodwin also noted that the bacterium possessed a unique ultrastructure as well as singular fatty acids that separate it from all other *Campylobacter* strains.

Its role as a pathogen secure, studies showing its association with various diseases continued to underscore the relevance of *C. pyloridis*. During a 12 month follow-up to eradication therapy for duodenal ulcers Coghlan et al. showed in 1987 that culturable *C. pyloridis* was a significant predictor for success or failure of treatment. 79% of patients who remained culture positive had a relapse, but only 27% of culture negative patients suffered the same fate. These findings were supported by Marshall 1988, Borody 1990 and Patchett 1992.
Gastritis-association was further corroborated by Hazell in 1987, when he found that the active chronic gastritis type B was limited to the antrum and the body of the stomach (reviewed in Marshall 1990).

In the meantime *Campylobacter pyloridis* underwent some changes in nomenclature. In 1987 its name was changed from the grammatically incorrect *Campylobacter pyloridis* to *Campylobacter pylori* (Marshall et al., 1987), then in 1989 there were considered to be enough singular features about it to create a new genus in its name: *Helicobacter*, from the Greek helical, as in spiral, and bacter, a staff, to describe its two most prominent features. *Campylobacter pylori* became *Helicobacter pylori*. The genus *Helicobacter* has since grown by adding novel species both in human and animal hosts.

The early nineties brought new evidence for old suspicions, gastric carcinoma and *Helicobacter pylori* were strongly associated in a study conducted on Japanese Americans in Hawaii (Nomura et al., 1991). Serum samples of 5908 men taken between 1967 and 1970 were tested for antibodies to *Helicobacter pylori* and correlated to the 109 cases of histologically confirmed gastric carcinoma. They found that even though the control group had 76% *Helicobacter pylori* positive sera the patients suffering from gastric carcinoma were 95% positive. These findings were mirrored in further studies (Forman et al., 1991; Parsonnet et al., 1991/1997).
In addition *Helicobacter pylori* has been associated with B-cell-lymphoma of the mucosa associated lymphoid tissue (MALT) indigenous to the intestinal tract (Wotherspoon et al.,

1991) and controversially even with ischemic heart disease (IHD) (Mendall et al., 1994; Pellicano et al., 1999).

It has been argued that eradication of *Helicobacter pylori* would lead to the increase of gastroesophageal reflux disease (GERD), basing this assumption on the observation that in the western world *Helicobacter pylori* infection is statistically sinking and GERD is on the increase (Blot et al., 1991). It has also been argued that infection with *CagA* positive strains of *Helicobacter pylori* reduced the risk of developing oesophageal and gastric cardial cancer (Chow WH et al. 1998). Both studies and others would imply that *Helicobacter pylori* was not simply a pathogen but also held a protective function in the human intestinal tract. This has been vehemently rejected by some authors (Axon, 1999) and been countered with the results of other studies such as a study done in Japan (Murai et al., 2000), where no increase of reflux oesophagitis, a prerequisite for oesophageal cancer, was found in *Helicobacter pylori* negative individuals.

2.2 What is *Helicobacter pylori*?

Helicobacter pylori is the best known member of the genus *Helicobacter* that consists of 18 species, only few of which are known to be human pathogens. They are Gram-negative, microaerophilic, flagellated and are either curved rods or in spiral form. Key features ascribed to the genus are, amongst others, the presence of sheathed flagella, a G+C content of the chromosomal DNA which averages at 35.2 mol% (Beji et al., 1988) and the existence of a glycocalix.

Helicobacter pylori is 0.5-0.9μm wide by 2-4μm long, has 4-6 unipolar flagella, a glycocalix roughly 40 nm thick (Goodwin et al., 1987/1990) and has its genome arranged in a single circular molecule that varies in size between 1.6 to 1.73 Mb (Beji et al., 1988).

In vivo *Helicobacter pylori* is actively motile, in culture it can undergo morphological change to coccoid form, which is associated with lowered cultivability and has controversially been argued to represent a dormant state as a reaction to a hostile environment (Bode et al., 1993; Nilius et al., 1993). Its metabolism appears to be respiratory (Mendz et al., 1991), but has yet to be completely elucidated.

Most notable biochemically is the abundant production of urease. This enzyme is initially intracellular and is later secreted by altruistic autolysis, an interesting mechanism in itself. When mucous gastric membranes are colonised, some of the bacteria die releasing urease and other outer membrane proteins (OMP) which then dissipate (Dunn et al., 1997), the transformation of urea to ammonia in the surrounding tissue providing an effective shield against gastric acid. This was proven in 1990 (Marshall et al., 1990) by exposing urease-negative bacteria and *Helicobacter pylori* to acidic environments in combination with urea. Only *Helicobacter pylori* survived. Therefore urease is an essential factor for the survival of *Helicobacter pylori* in the strongly acidic environment of the stomach.

2.3 Genetics

Although 40% of isolated *Helicobacter pylori* contain one or more plasmids, these do not appear to encode any virulence factors (Kleanthous et al., 1991; Minnis et al., 1995).

Genetic variation on all levels of the genome is well documented (Jiang et al., 1996). It displays an apparently endless macro- and microdiversity, while the phenotype itself remains relatively stable.

Comparison of the genetic maps of five *Helicobacter pylori* strains demonstrated that there is no characteristic arrangement or clustering of 17 known genes (Jiang et al., 1996). This diversity is underscored by the fact that while *VacA* and others are always present, many genes, among them such markers of pathogenicity as *CagA*, can be present or not (Cover, 1997; Murali et al., 1993; Censini et al., 1993).

Both these genes are good examples of the amount of alleles found to be in existence during *Helicobacter pylori* research. When *CagA* was first described (Censini et al., 1993; Murali et al., 1993), one of the questions sought to be answered was whether the similar but inconsistently built genes associated with pathogenicity could effectively be grouped into one. Both research teams succeeded in doing so, showing that *CagA* had many alleles. The acronym stands for 'cytotoxin associated gene', for even though *CagA* is secreted into the host, they did not find the product to be toxic in itself. It has however been shown that it is frequently associated with diseases such as gastric- and duodenal ulcers and gastric cancer.

VacA stands for 'vacuolating cytotoxin', for its product induces vacuolisation in gastric epithelial cells (Cover et al., 1997), which may ultimately lead to ulcers. This gene exhibits so called mosaicism, having to date four different families (s1a, s1b, s1c, s2) and two different families of middle-region alleles (m1, m2) (Cover et al., 1997). The genetic pattern of the *VacA* gene has been correlated with the presence of various illnesses, as demonstrated in various studies in different countries (Atherton et al., 1997; Letley et al., 1998; Evans et al., 1998; van Doorn et al., 1998). They also showed that different alleles of *VacA* seem to have different effects on the population of different countries.

The arrangement of genes of five isolates mapped by PFGE (pulsed field gel electrophoresis) have been analysed by Jiang (Jiang et al., 1996) revealing that there is little conservation of gene location among the strains. This is in marked contrast to other bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Krawiec et al., 1990).

To date the genomes of two strains of *Helicobacter pylori* have been sequenced in their entirety and an analysis as to their similarity was published in 1999 (Alm et al., 1999).

According to this paper the overall diversity in *Helicobacter pylori* has been misinterpreted due to an unavailability of two complete strains, stating that only 6-7% of genes are specific to each strain and almost half of those genes being clustered in a single hypervariable region. Comparing its results to *Mycobacterium tuberculosis* (Fleischmann RD et al., published 2002) the authors can however determine a nucleotide polymorphism in *Helicobacter pylori* two to three orders of magnitude higher than that of *Mycobacterium tuberculosis*, again upholding its immense variability.

In population genetic studies (Achtman et al., 1999; Suerbaum et al., 1998) it has been shown that strain diversity in *Helicobacter pylori* has arisen as a result of recombination between strains and that new recombinant genotypes continue to be generated in patients infected with multiple strains. This was further demonstrated by Kersulyte et al., 1999. In a Lithuanian patient with a proven long-term infection with two parent strains six different variations of the two could be found using PCR, hybridization and DNA sequence-based evidence.

2.4 Epidemiology

Helicobacter pylori has been found in the stomachs of humans the world over and is one of the most common pathogens known to man. In developing countries large sections of the population can be infected, despite which the disease to infection ratio stays quite small: Research conducted on 177 *Helicobacter pylori* positive male subjects in India shows a point-prevalence of 7.8% active ulcer disease (Khuroo et al., 1989), similar numbers are reported in other articles. This relatively low number must be seen in the relation of prevalence, which is exceedingly high.

Factors promoting infection with *Helicobacter pylori*, so-called risk factors, are much sought after and many have been promoted. The most important and almost universally confirmed risk factor in the acquisition of *Helicobacter pylori* is low socio-economic status. In a recent sero-epidemiological study of 11'605 sera collected from all age groups and social classes in Mexico, Torres (Torres et al., 1998) could confirm these findings. A co-twin study (Malaty et al., 1999) could demonstrate that hygiene standards rather than genetic profiles of carriers were responsible for infection. Other risk factors include large families (Kikuchi et al., 1998) and parents history of gastric disease (Brenner et al., 1998). More controversial discussion has been held on the subject of gender and race. Male gender seems to be a significant risk factor in western countries (Lin et al., 1998). Afro-American children suffered more infections with *Helicobacter pylori* over a study interval of 12 years than a group of Anglo-Americans observed in the same community for the same period of time (Malaty et al., 1999).

Prevalence can range from few percentage points to major parts of the population, even within single countries there are large variations. England has many communities of predominantly immigrant or native inhabitants. In an interesting study conducted in central England southern Asian immigrants and their children born in England were found to have a significantly higher rate of infection (47%) than a similar control group of native English people (15%). It can be postulated that *Helicobacter pylori* is transmitted in a family setting and that following that logic families would all be infected with similar strains of the bacteria.

Transmission of *Helicobacter pylori* has been studied extensively, but no conclusive results have been found. As it has been isolated from animals such as cats (Neiger et al., 1998), contamination via animal spreading has been discussed. Hypothesizing that common houseflies have contact with human excretions and have been proven to transmit other diseases a study performed in Boston (Grubel et al., 1997) was successful in culturing *Helicobacter pylori* from both the surface and the gut of flies that were exposed to it after a controlled period of time. Evidence of *Helicobacter pylori* by PCR amplification has been

found in water, food and soil, but no culture has ever been successful using these as inoculation material. Consequently, the most popular theory is that it is transmitted gastro-orally. Luzza (Luzza et al., 2000) proposes that *Helicobacter pylori* is transmitted in a family situation through vomiting of smaller children and contamination of others. Children were recruited and selected according to a so-called vomiting index. Results show that siblings of children with a high vomiting index have an increased risk of suffering from active infection. This would also help to explain the early age of infection that has been reported in developing countries (Rothenbacher et al., 2000).

2.5 Strain typing

The methods used in molecular genetics have handed medical researchers a powerful tool in their quest to cure disease. Genetic fingerprinting is the most reliable way of identifying a pathogen, be it bacterial, viral or inherited, and can be of invaluable help in epidemiological study, creating new diagnostic approaches and furthering the development of new treatments.
The idea is to access a pathogen's genome in such a way that each can be distinguished from even its closest relative. A selection of the most common methods is given in **Table 1** in the appendix section.
The method of choice for us was the amplification of genes using the "polymerase chain reaction" with following restriction using "random fragment length polymorphism" (PCR-RFLP). In 1996 Kansau et al. found it to be superior to the RAPD (random amplified polymorphic DNA) technique when they tested patients with gastritis before treatment and after unsuccessful treatment or relapse. It had the advantage of being selective in the section of gene targeted and being more easily reproducible.
Burucoa (Burucoa et al., 1999) performed a combination analysis using experimental results as well as raw-data from literature and performed a meta-analysis of PCR-RFLP, ribotyping and RAPD. Concerning discriminatory power and typeability, the ability to give unequivocal results on analysis, PCR-RFLP succeeded in surpassing both remaining candidates. It was therefore proposed that PCR-RFLP should be the method of choice for the DNA-based differentiation of *Helicobacter pylori*.
An interesting question was posed in a prospective study by Jeen (Jeen et al., 2001). After identical treatment with triple therapy some dyspepsia patients experience relapse. In this study, 10 patients were chosen with relapse after 6-24 months, another 10 patients with unsuccessful treatment and relapse on cessation of therapy were added as a control. In order to differentiate between recurrence of previous infection and re-infection with an alternate strain of *Helicobacter pylori* PCR-RFLP was used on biopsy samples from both pre- and post-treatment gastroscopies. Where most strains were identical in both pre- and post-therapy samples, the group with relapse after 6-24 months showed a higher incidence of infection with new strains as differentiated by PCR-RFLP.
PCR-RFLP uses so-called profiles, describing both the segment of the genome targeted in the PCR amplification process and the restriction enzyme used in the following analysis. Owen (Owen et al., 1998) used an *UreAB*/*NdeII* profile in order to separate 383 samples obtained from 10 different countries. Using this profile they defined 82 different RFLP patterns of

which the ten most common patterns accounted for 51% of all samples. Only 18% of the samples had unique RFLP patterns leaving a vast amount of multiple band patterns from strains harvested from unrelated patients. Some of the sample bacteria that were considered to be genetically identical exhibited different levels of virulence in vivo. Maybe there is some other factor influencing those supposedly identical organisms, something in the patients alimentary habits, smoking, weight, genetic constitution, etc.. Maybe, however, the organisms may only seem to be identical in the one fingerprint that was taken. In a bacterium as genetically diverse as *Helicobacter pylori* this of course begs the question; Is there such a large amount of genetic homogeny or is the test not precise enough? This was what we wanted to put to the test, our reasoning being that additional testing was required if one set of PCR-RFLP presented us with data that described strains as identical that showed different levels of in-vivo virulence. If one set of fingerprinting with one gene allows a certain amount of discrimination then a combination with a second set of fingerprinting using a second gene should result in higher differentiation.

Typing in itself is work-intensive and the information gleaned from it must be transferred to paper or be digitized for the sake of preserving it since the agarose gels can not be stored for any length of time. A database-program such as GelComparII (Applied Maths, Belgium) is an ideal way of storing not only the derived data from a PCR-RFLP-run but the data itself by digitizing the band patterns and sorting them by strain. This has not been widely performed or at least published, a Medline search revealed only 22 results to the search term GelComparII. A study done by Kivi (Kivi et al., 2003) on the concordance of *Helicobacter pylori* strains within families used GelComparII to map the interrelatedness of the different strains obtained by different family members. They showed that GelComparII defined roughly 25% more strains as singular than visual inspection, emphasizing that visual inspection of strain definition and correction of automatically assigned bands was essential even after utilizing all normalization and filtering techniques available in the program. The importance of visual inspection of all digitized and computer-sorted data is seconded in an article comparing two different database and interpretation programs done by Gerner-Smidt (Gerner-Smidt et al., 1998) stating that both programs, though performing well, require the user to make critical decisions in the analysis.

2.6 Synopsis

Helicobacter pylori is one of the most common pathogenic bacteria in the human body and has been found around the world. Its abundance and its association with important and widespread illnesses has been demonstrated. Research into this pathogen must be pursued if eradication or at least management is to be achieved. This is even more important since treatment will not be able to be effected via widespread use of antibiotics indefinitely, sooner or later other methods of treatment will have to be available to counter resistance developed in such a versatile organism. Antibiotics being costly current treatment is not an option for developing nations that have to harshly limit expenditure on the medical sector. Alternative treatment strategies will be essential if science wants to be fair to all nations.

Understanding more clearly which organisms have which effect on the human body is essential for progress in this area. Fingerprint typing using PCR-RFLP is a viable and reproducible way of differentiation between strains as well as establishing links between phenotype and genotype, as has been demonstrated in many studies.

Typing of *Helicobacter pylori* strains and classification according to virulence would offer physicians worldwide an alternative to simply treating every case of *Helicobacter pylori* infection as is standard practice today. They could choose to treat only virulent strains antibiotically, which would again reduce the amount of resistance developed by the bacteria and facilitate treatment if it was necessary. Later on, this list of virulent strains could be adapted for any population or race, if necessary.

3. Objective

The reason the research in this paper was performed was to find an efficient and practicable method of genetically differentiating *Helicobacter pylori* strains found in gastric biopsy samples.

Of the previously described methods PCR-RFLP was selected as the most viable test as it has been proven to be effective in differentiation in many studies and has the advantage of being reliably reproducible.

The objective of this paper is to prove that two PCR-RFLP's using two separate genes are superior to the single run in differentiation.

4. Materials and methods

The *Helicobacter pylori* strains used in this study were isolated from tissue samples of patient gastric tissue harvested in biopsy form during endoscopic procedures in the University Hospital of Zürich, Switzerland, between 1997 and 1998 (PD Dr. med. P. Bauerfeind).

4.1 Culturing

Half of each sample was spread onto Wilkins-Chalgren-Agar (Life Technologies) containing both Skirrow's antibiotics (Vancomycin 10 mg/l, Cefsulodin 2 mg/l, Trimethoprim 5 mg/l, Actidion 100 mg/l) and 5% sheep-blood (BAG Med. AG, CH), while the other half was stored at -70°C. The plates were placed in an incubator at 37°C in a microaerophilic, water-saturated atmosphere (created by CampyPak, Becton Dickinson) for 3 – 4 days, then checked for growth. If growth was present the clusters were subcultured onto the same media to increase bacterial yield and eliminate contaminants, if not, they were left for an additional two days. When ready, bacteria were harvested and collected in 500 µl of TE buffer (50 mM Tris HCl, pH 8.0, 1 mM EDTA). This mixture was suspended by vortexing.

4.2 Phenol/Chlorophorm extraction

Cells were harvested in a table top centrifuge at 8000 rpm at room temperature for 5 minutes, the supernatant poured off and another 500 µl of TE buffer added before resuspension by vortexing. 20 µl of lysozyme (1 mg/ml H_20, Boehringer Mannheim) was then added and the mix incubated for 30 minutes at 37°C. After further addition of 5 µl of proteinase K (20mg/ml, Boehringer Mannheim) solution, 30 µl of 10% SDS (Sodium Dodecyl Sulphate) and 45 µl of TE buffer we repeated the incubation at 37°C for 2.5 hours. 100 µl of NaCl (5M) and 80 µl of pre-warmed CTAB (Cetyl tri methyl ammonium bromide, 10% in 0,7 M NaCl, SIGMA) were then added before further incubation at 65°C for 10 minutes, after which 780 µl of chloroform/isoamyl alcohol (SIGMA) were mixed in by slow inversion. Centrifugation (Biofuge fresco, Heraeus) followed at 14'000 rpm for 10-15 minutes at 4°C, then the supernatant was carefully aspirated. The procedure was repeated with 750 µl of phenol/chlorophorm (SIGMA), supernatant again aspirated carefully and 640ml of isopropanol (SIGMA) added. After gentle mixing the sample was stored at –20°C for two hours. Centrifugation at 14'000 rpm for 20-30 minutes at room temperature was the next step

in order to precipitate the DNA, which was thereafter washed with the same volume of 70% ethanol under the same conditions. In order to dry the DNA pellet, the samples were placed in an exsiccator connected to a vacuum pump (KNF Neuberger Lavoport Membranvakuumpumpe) for 10-15 minutes. Finally 50 µl of TE buffer were added to DNA for conservation purposes.

4.3 PCR amplification

UreAB and *UreCD* were chosen as target genes because they are found in every strain of *Helicobacter pylori*.
The primers used are listed in **Table 2** in the appendix section.

Amplification of *UreAB* was carried out in 100 µl using 2.5 µl template DNA from *Helicobacter pylori* isolates, 2 x 2 µl of each primer (100 nmol/ml, Microsynth CH), 2 µl dNTP (10 mM each, Boehringer Mannheim), 2 µl hot-start taq-polymerase (1 U / µl, QIAGEN), 8 µl $MgCl_2$ (25 mM stock solution, QIAGEN), 10 µl buffer (25 mM, Boehringer Mannheim) and 71.5 µl of sterile Millipore water. For *UreC-UreD* 4 µl of $MgCl_2$ was found to be sufficient for optimal amplification, which brought the amount of distilled water up to 75.5µl to account for the difference in volume. PCR was performed in a Techne Progene PCR cycler (Witec AG, CH). Details of protocol are given in **Table 3** in the appendix section.

The resulting product was tested by running an agarose-gel electrophoresis. A 1.2% agarose gel was created by using Life Technologies agarose and a 10 fold concentrated TPE buffer (1 litre contained 108.0g of Trisbase (SIGMA), 29.78g of Na_2-EDTA $2H_2O$ (SIGMA), sterile water and the pH was brought to 7.6 using the appropriate amount of H_3PO_4 (MERCK)). The gel was poured into a 7x11 cm well, left to set and then immersed in the same TPE buffer, three wells were filled with 25 µl of each product (with the addition of 5 µl of loading buffer (33% glycerine, 7% SDS, 0.07% bromophenolblue in sterile water)). After running, staining with ethidiumbromide (10µg/ml) and viewing in a Vilber Lauromat UV transilluminator (wavelength 254 nm) the relevant bands were excised for further processing.

4.4 DNA extraction from Gel

DNA was extracted from the gel using the QIAamp Gel Extraction Kit (QIAGEN AG, CH). In compliance with the manufacturers manual TE buffer was added to the gel-bound excised bands and were spun for 15 mins at 4°C with 13‘000 rpm. All fluid was poured off, leaving only a solid pellet at the bottom of the vial. 100 µl of ATL buffer as well as 40 µl of proteinase K stock solution (20 mg/ml) were added and the mix incubated at 55°C overnight in an Eppendorf thermomixer (400 rpm). 20 µl of RNase A (20mg/ml, Boehringer Mannheim) was added the following day to rid the mix of possible RNA-contamination, the vial was then vortexed and incubated at room temperature for 2 minutes. Addition of 200 µl of buffer AL, vortexing and incubation at 70°C for 10 minutes followed, whereupon 210 µl of >98% ethanol were added before repeated vortexing. The QIAamp spin columns were filled with the mixture and, with a 2 ml collection tube as receptacle. All of the following centrifugation steps were performed at room temperature. Centrifugation at 8000 rpm for 1 minute was performed (Heraeus Biofugue tabletop cooled centrifuge). The QIAamp column was placed in a fresh collection tube, 500 µl of buffer AW poured into the spin column and centrifugation repeated at 8000 rpm for 1 minute. A new collection tube was used and 500 µl of buffer AW placed in the spin column once again and the mix centrifuged for three minutes at 13'000 rpm. Then the QIAamp spin column with the extracted DNA was placed into a clean 1.5 ml (or 2 ml) microfuge tube, 210 µl of buffer EB heated to boiling point and 100 µl of it added to the QIAamp column. Centrifugation was done at 8000 rpm for 1 min, after which the process was repeated. The DNA was then ready to be precipitated from the buffer EB. The mix was transferred to a 1.5 ml Eppendorf cup and 3 M sodium acetate (Boehringer Mannheim) – 10% of the mix volume – and twice the mix-volume of 100% absolute alcohol (SIGMA) were added, the resulting solution stored at -4°C overnight. The next morning all samples were centrifuged with 13'000 rpm at 4°C, the remaining fluid was discarded and the pellets dried in a vacuum for 10 minutes, before being redissolved in 24 µl of sterile H_2O. This mix was the basis for digestion.

4.5 Restriction of PCR products

UreAB was digested using *HaeIII* (Boehringer Mannheim, 10U/µl). 1 µl of *HaeIII* was added to 10 µl of PCR-product together with 1.2 µl of the provided optimal reaction buffer M. This mix was left at 37°C overnight in a theramablock (Eppendorf Thermomixer comfort). The

following day electrophoresis was performed using a 2% agarose gel in order to separate the resulting restriction bands. These gels were stained using ethidium bromide and Polaroid photographs were taken. *UreCD* was digested using 0.5 µl *NdeII* (5U/µl, Boehringer Mannheim) and 10 µl of the provided special buffer to 10 µl of the PCR-product. The rest of the procedure was the same as with *UreAB*.

4.6 Evaluation of gel patterns

All Polaroid pictures of gels were analysed conventionally in comparison to the standardised bands that were also run with the samples during testing. An example of the Polaroid results can be seen in **Figure 1** in the appendix section. The base-pair sizes of each fragment after both PCR- and restriction-runs were entered into **Table 2** and **Table 3** and can also be seen in the appendix section.

The Polaroid pictures were scanned (**Figure 1**) for evaluation in Applied Maths' (Belgium) GelComparII program. The bands corresponding to a biopsy sample were electronically separated from the gel it was run on (**Figure 2**) and a database built from individual band-structures (**Figure 3**). The bands on the patterns are then marked automatically by the computer program and their size correlated to standard size bands that ran with the corresponding gel in order to account for inequalities in gel structure and act as reference points. After calibration the relationship between the band patterns can be assessed and are graphically displayed (**Figure 4**).

This was done for both PCR-RFLP runs and the two graphs were then compared as further described in the results section.

5. Results

Of the 37 frozen biopsies from unrelated patients 33 samples were successfully cultured, 4 resisted culturing. All 33 samples allowed amplification and restriction of both the *UreAB* and the *UreCD* segment of the genome with *HaeIII* and *NdeII* respectively.

Two different analyses of the Polaroid pictures of the ethidium bromide stained electrophoresis gels (samples of which are pictured in **Figure 7** and **Figure 8**) were performed, both a conventional visual approach and a computerized assessment of interrelatedness using GelComparII (Applied Maths, Belgium).

The results of visual analysis have been grouped in **Table 4** and **Table 5**.
The *UreAB/HaeIII* restriction yielded a total amount of 20 band patterns with 10 patterns encompassing 2 or more stains. 10 strains were found to be singular.
UreCD/NdeII was less discriminatory in this experiment showing only 14 band patterns in total with 4 band patterns having 2 or more samples attributed to them. As in the *UreAB/HaeIII* run 10 singular band patterns were found (**Table 7**).
Combining the results of both the *UreAB/HaeIII* and the *UreCD/NdeII* runs leaves two strains, strains 4 and 37 identical in both runs (**Table 6**), all other strains could be clearly defined into singular organisms. **Table 6** also shows the pattern-partners of each strain in all four variants of analysis.

UreAB/HaeIII as analyzed by GelComparII shows a higher differentiation into a total of 30 band patterns with only 2 patterns with 2 or more samples attributed to them.
As expected from conventional visual analysis *UreCD/NdeII* is less discriminatory showing a total of 23 band patterns and 5 pattern with 2 or more strains attributed to them (**Table 7**).
Combining the two PCR-RFLP runs leaves no strain grouped to another, in effect all strains have been defined as singular.

In the *UreCD/NdeII* run GelComparII shows 5 band patterns with 2 or more strains attributed to them whereas the visual analysis shows only 4. This is attributable to the fact that a strain defined as singular in the visual analysis (strain 2) is grouped to another strain (strain 22) which, contrary to visual analysis, is not grouped in the largest of multiple strain patterns.

This leads to an additional subgroup and a higher number of strain patterns with 2 or more strains.

With this exception all strains both in the *UreAB/HaeIII* and the *UreCD/NdeII* PCR-RFLP runs are consistently grouped into the same band patterns both in visual analysis and in GelComparII interrelatedness assay (**Table 6**). The large difference in the amount of band patterns between visual and GelComparII analysis is due to GelComparII defining more strains as singular than was considered correct during visual assessment.

When on final analysis all results are pooled more weight was given to visual analysis than GelComparII as advised by literature (Gerner-Smidt et al., 1998) and two strains (strains 4 and 37 as marked in **Table 6**) remained identical in both *UreAB/HaeIII* and *UreCD/NdeII* PCR-RFLP runs and were therefore considered to be the same strain of *Helicobacter pylori*.

The sum of the base-pair (bp) sizes of the restriction bands of an individual restriction run are expected to correspond to the total bp size of the section of Genome in question. In *UreAB/HaeIII* the sum was smaller than expected in 2 strains (3 and 10) and larger in 3 (20, 26 and 33). *UreCD/NdeII* also showed 2 smaller than expected total bp counts, strains 24 and 36, 2 strains were larger than expected (26 and 33) (**Table 4** and **5**).
Only samples 26 and 33 were larger than expected in both restrictions, all smaller than expected samples were unique to one restriction run. These two samples are hypothesized to contain two separate strains of *Helicobacter pylori*.

During the normalisation routine while digitizing the Polaroid pictures of PCR-RFLP patterns into GelComparII much care was taken to correct the automatically assigned bands using both the Polaroids themselves and the previously performed visual analysis. After normalisation all patterns looked visually identical, no unexpected bands could be newly defined. The difference in evaluation using GelComparII or visual approach derives purely from slight variations in estimation of individual bp sizes of the bands.

6. Discussion

The success of this experiment is the fact that almost complete differentiation of every patient sample into a singular strain could be performed using two gene loci for PCR and an individual restriction protocol with visualisation using gel electrophoresis. Using either of the two amplification-restriction protocols was not sufficient, both amplification-restriction protocols had to be combined as described in the results section. As all patients were unrelated and did not know each other this could be expected and would be consistent with the genetic variability of *Helicobacter pylori*. The one remaining pair of strains (numbers 4 and 37 as marked in **Table 6**) identical in both restrictions is acceptable and also to be expected, as identical strains are bound to exist following transmission from one person to another.

The two analyses of the *UreAB/HaeIII* and *UreCD/NdeII* protocols, i.e. manual/visual and with the GelComparII software, differed in result most notably in that GelComparII did not find strains 4 and 37 to be identical. Whereas manual evaluation always gives space for and necessitates interpretation of results at every level, defining band sizes, lenience for electrophoresis-gel-warping, etc., GelComparII is much more rigid. After scanning the bands some correction for gel warping can be performed and some correction of the automatic allocation of bands can be done (Gerner-Smidt et al., 1998). These are by definition manual steps and have the same inherent faults as pure visual evaluation. Where GelComparII becomes more rigid than visual evaluation is in the definition of levels of interrelatedness of the strains; after exact definition of each band of each strain there is little allowance for gross similarity, the patterns are grouped according to strict protocol. This protocol can be adapted with the addition of certain lenience percentages to correct for obvious mistakes, but is the inherent difference in the two evaluation methods.

This explains why the GelComparII results in **Tables 6** and **7** show more unique band patterns. The obvious advantage to GelComparII is that it allows the comparison of hundreds or even thousands of band patterns and the building of intra- and inter-laboratory databases.

In keeping with literature (Kivi et al., 2003) visual evaluation was taken as the gold standard and results were interpreted accordingly.

After adding all bands of each strain they were compared to the expected size of the *UreAB* or *UreCD* loci as a control feature. As described in the results chapter some were smaller and some larger than expected. The differences in size that were only registered in one of the two

runs were considered to be erroneous, an error in measuring or evaluation must have occurred that led to the deletion or addition of bands. Another explanation for the size-difference could be double-bands where two fragments of almost equal size appear to be hidden in one appearing 'thicker' (more fluorescent) than normal. This would be very difficult to detect in the current experimental setting.
Two strains, numbers 26 and 33, that were larger than expected in both PCR-RFLP runs remained. These two patients were considered to be infected with two different strains of *Helicobacter pylori* showing some overlap in band-size which accounts for the fact that the expected total size of the genes is not double the norm. Colonization of individuals with more than one strain of *Helicobacter pylori* appears to be rare among Europeans and North Americans. Taylor (Taylor et al., 1995) found more than one colonizing strain in only three out of 15 patients and Marshall (Marshall, 1996) found only one out of 19 patients to be infected with two strains. In sharp contrast developing countries such as Peru can show a double infection rate of up to 34% (Berg et al., 1997).

As does any method of research our applied method of culturing with following PCR-RFLP has its disadvantages. The most obvious ones would be faults in transportation, handling and storage as well as human error in all steps of analysis. The most notable weaknesses can be differentiated into two sections; culturing, not dependent on skill, where 4 samples were lost due to no growth, and the analysis of the Polaroid pictures of the electrophoresis gels which is very dependent on skill and experience. Some variation in thickness in the gels used to separate the bands electrophoretically is to be expected. This leads to warping of the band patterns of the restricted PCR products which can at times become rather extensive. It can be countered by repeated running of PCR-RFLP products which is very time- and cost- intensive in both equipment and manpower or, as performed here, with the addition of a computer program such as GelComparII which has an inbuilt function to straighten the bands according to the simultaneously run standardised bands as described in the results section. All these correction methods are however at times arbitrary and rely heavily on the experience and precision of the researcher.

In an insightful article in Science magazine Daniel Falush et al. (Science 2003) recently harvested 370 strains from 27 geographical and ethnic human groupings and using the sequenced information of seven housekeeping genes, one virulence associated one (*VacA*) and a computer program called 'structure' presented a history of human and *Helicobacter pylori*

migration. The program analysed all sequenced bacteria and grouped them into four modern populations, which were then subdivided into a total of seven major populations. It could be proven that *Helicobacter pylori* has been associated with humans for such a long time that the interrelatedness of the strains corresponds to the migration of humans from continent to continent up to and including the slave-trade from Africa to America and Europe.

This experiment would like to be seen in the light of continuing important research into the nature of *Helicobacter pylori*. We set out to prove that the combination of multiple PCR-RFLP's with multiple genes was superior to the single protocol performed on any one strain. The breaking up of the grouped strains by the second PCR-RFLP into new groups or singular profiles, as shown in **Table 6**, resulted in a markedly superior differentiation of strains. In consequence we propose that no analysis of *Helicobacter pylori* should be based on one method of differentiation alone. An ideal combination of tests would be capable of definitively differentiating between two strains of *Helicobacter pylori*. If this idea would be extrapolated to its maximum potential a database of the genotypes of known strains could be correlated with the phenotype, i.e. the virulence of the strain itself. This would enable the precise administration of antibiotic drugs where necessary and would help curtail both the development of resistance towards these medications and reduce the costs to society, therefore increasing the population that could afford to get effective treatment for their illness.

7. Appendix

Table 1

Short introduction to selected methods of genetic differentiation (so-called fingerprinting)

TECHNIQUE	EXPLANATION
Culture	Mixtures of bacteria are reared on media that select for a specific bacteria. As they each have their own way of metabolising substances they need, have their own favourite culture conditions and show differences in their resistance to antibiotics, separation of bacteria can be achieved through the choice of supplied nutrients, given pH-value or other conceivable valuables (Hachem et al., 1995) added to the media the bacteria is allowed to grow on. It is very cost- and time-intensive as it requires incubators, specific media and surrounding atmospheres and time to await results as well as personnel to run the laboratory. There is only limited possibility for separating one strain of bacteria from another, such as when alleles encode for basic functions. Though not strictly speaking a method of genetic fingerprinting, it is widely used for differentiation of known organisms on the species level and is mentioned in that light.
PCR (polymerase chain reaction)	PCR is widely used in the scientific community as a method for amplifying defined sections of DNA. If for example a single known gene is to be amplified by a pair of primers, single stranded inverse copies of the initial sequences of the target gene, as read on both complementary sides of the DNA-strand in question, also known as the template, is produced to act as a key. The primers are added to a mixture of heat-resistant DNA-polymerase, nucleotides, buffer and Magnesium. This mixture is then heated in order to separate the double-stranded DNA. During the cooling process that follows, the primer is able to anneal with the appropriate complimentary sequence of the gene, the polymerase picks up where the primer ends and extends the primers with the provided nucleotides; the gene is replicated. After renewed heating the copy is separated from the original single strand and the amount of gene available has been doubled. These steps are called one cycle and after many cycles the gene in question has been amplified exponentially. PCR is capable of amplifying a single gene to such numbers that testing becomes possible and has therefore become indispensable in the world of genetics.
PFGE (pulsed-field-gel-electrophoresis)	A section of DNA is digested by restriction endonucleases, enzymes that can recognise certain successions of nucleic acids within which they cut the strand. PFGE allows the resulting DNA fragments to be separated by the inherent negative charge of the phosphate groups. The samples are placed in little slots in agarose-gel (or any other kind of gel), the gel immersed in a buffer fluid and an electric current applied in such a way that the nucleic acid strands migrate through the gel. The mesh-like structure of the gels allows their passage, whereby larger strands travel slower than shorter ones separating them according to size. The resulting patterns can be visualised using ethidium bromide or other nucleic acid dyes (Jiang et al., 1996).
Ribotyping	Ribosomal RNA-molecules (rRNA) are found universally and exhibit a highly conserved structure. This method requires an organisms strands of nucleic acids, both chromosomal and ribosomal, to be digested by nucleases and the resultant to be separated by PFGE. The resultant gels can be hybridised using radioactively marked DNA or RNA probes, depending on the questions to be asked Phylogenetic analysis and the creation of orders of bacteria has been one of the major fields of its implementation. It has also been successfully used in a clinical setting to type the *Helicobacter pylori* strains of pre- and post-therapy non-responders, which were found to be identical (Rautelin et al., 1994).
RFLP (restricted fragment length polymorphism)	Chromosomal DNA is digested by restriction endonucleases and separated by fragment size via gel electrophoresis. The resulting fragments are hybridized with DNA probes called primers made from known genes carrying a marker. During hybridization the primers bind to their genetic counterpart and allow further differentiation by use of their attached marker. This method has been used to construct a genetic map for *Helicobacter pylori*, which turned out to be a good example for the large inner-species variability at a genetic level (Taylor et al., 1992).
PCR-RFLP	A specific gene is amplified using PCR. The resulting yield is digested by restriction endonucleases and the fragments separated by gel electrophoresis. This method enables research into allelic variation, which is one of the keys to differentiation. PCR-RFLP has been proven to be effective in typing strains and provides a reliable test for future reference (Akopyanz et al., 1992, Foxall et al., 1992).
RAPD (random amplified polymorphic DNA)	Short single primers are added to a PCR mixture and PCR is performed. As opposed to PCR-RFLP this does not result in a specific and continuous product, random annealing at corresponding areas of the genome occurs and creates a number of variously sized fragments. These are however specific to a strain of bacteria. Because the methodology relies on the PCR creating many different fractions that would enable differentiation size becomes critical. RAPD has been used successfully in distinguishing between clinical isolates (Akopyanz et al. 1992).

Direct PCR	The biopsy samples have their DNA extracted directly, without any culturing of bacteria. This method is highly sensitive but requires a very well thought-out and tested PCR protocol because of the amount of interfering DNA contained in the sample. Hennig et al. examined in 1999 the presence of *CagA* and *VacA* in patients with either duodenal ulcer (DU) or gastritis (G). They found a definite positive association for *CagA* and the s1 genotype of the *VacA* gene with DU patients.
Sequencing of genome	DNA is subjected to a treatment similar to PCR. The same sequence of heating, annealing and replication is performed with the difference that the normal nucleotides are exchanged for altered ones that, when incorporated into a strand halt production. All four "defective" nucleotide types are labelled and can be recognised by the wavelength of the light they emit when subjected to laser lighting. All strands will have very different lengths and their respective ends will be the only thing labelled. By separating them electrophoretically on a polyacrylamid-gel the exact position in the strand can be found and all positions can be traced. Knowing the exact sequence of a genetic strand is the ultimate information gainable for genetic research. This is however immensely time- and cost-intensive and not feasible in everyday research. To date only two strains of *H. pylori* have been sequenced completely (Alm RA et al. 99 Nature) and surprisingly showed a large overall genetic similarity.
PCR-DNA sequence typing	A specific gene is amplified via PCR and then typed by direct DNA sequencing. If the chosen gene was a very common gene, such as in the case of *Helicobacter pylori* the one for urease, it could be sequenced for all known strains and biopsy bacteria could be correlated with a database built from this knowledge (Atherton et al., 1995).

Table 2

UreA-UreB		
Sense	5'- AGG AGA ATG AGA TGA –3'	
antisense	5'- ACT TTA TTG GCT GGT –3'	
Expected fragment size 2.4 kb		Foxall et al., 1992
UreC-UreD		
Sense	5'- TGG GAC TGA TGG CGT GAG GG –3'	
antisense	5'- ATC ATG ACA TCA GCG AAG TTA AAA ATG G –3'	
Expected fragment size 1.7 kb		Labigne et al., 1991

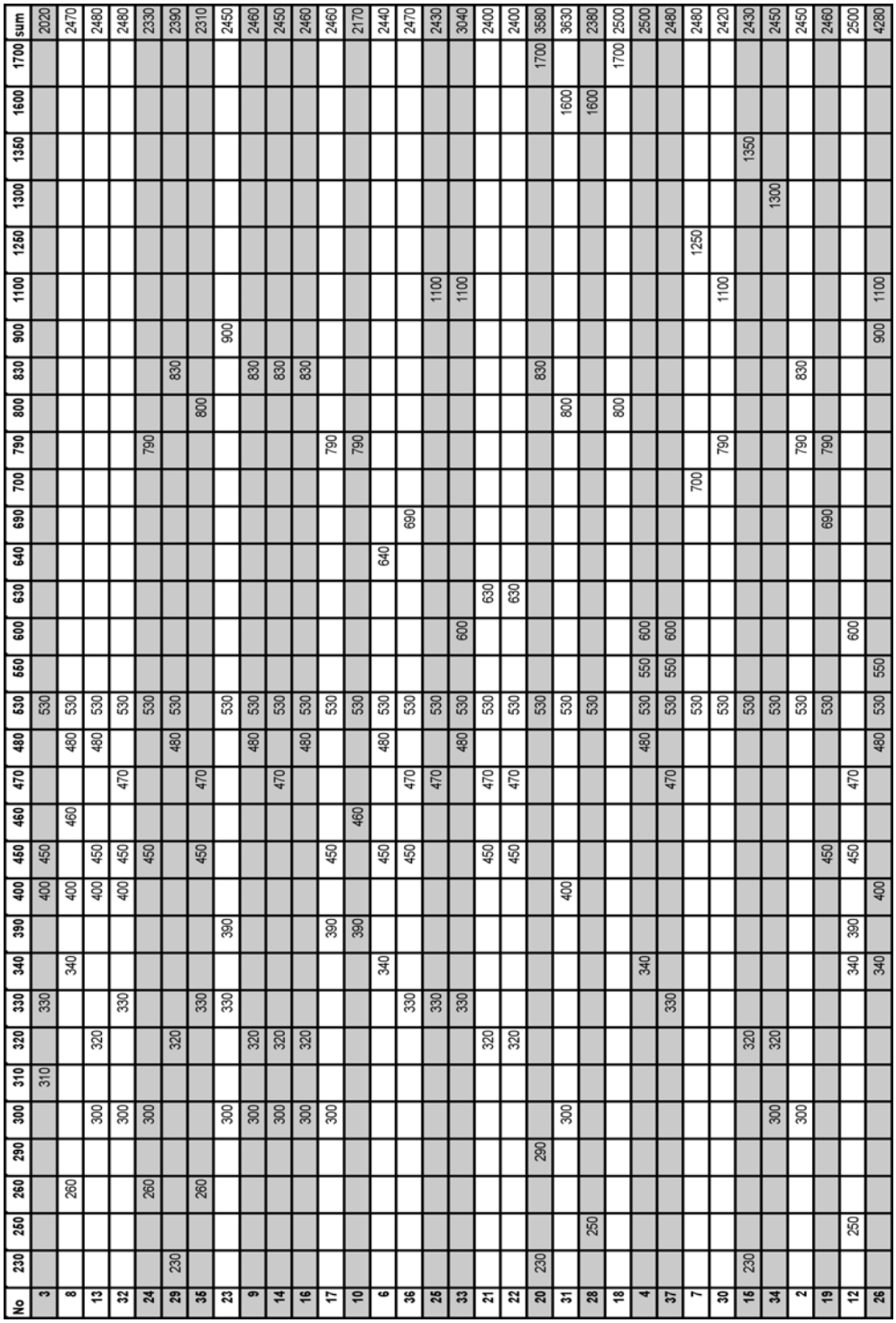
Primers used for amplification

Table 3

PCR cycling protocol used for amplification of both *UreAB* and *UreCD*

1. cycle	1.segment	95°C	for 5 mins	
	2. segment	85°C	for 1 min	Stop to add taq
2. cycle	1. segment	94°C	for 1 min	
	2. segment	45 / 60°C	for 1 min	repeat cycle 2. x 35
	3. segment	72°C	for 2.5 / 1.5 mins	
3. cycle		72°C	for 10 mins	
4. cycle		4°C	Loop	End of protocol

Table 4



No	230	250	260	290	300	310	320	330	340	390	400	450	460	470	480	530	550	600	630	640	690	700	790	800	830	900	1100	1260	1300	1360	1600	1700	sum
3						310		330			400	450				530																	2020
8			260						340		400		460		480	530																	2470
13					300		320				400	450			480	530																	2480
32					300			330			400	450		470		530																	2480
24			260		300							450				530							790				1100						2330
29	230						320								480	530									830								2390
35			260					330				450		470										800									2310
23					300			330		390						530										900							2450
9					300		320								480	530									830								2460
14					300		320							470		530									830								2450
16					300		320								480	530									830								2460
17					300					390		450				530							790										2460
10										390			460			530							790										2170
6									340			450			480	530				640													2440
36								330				450		470		530					690												2470
25								330						470		530											1100						2430
33								330							480	530		600									1100						3040
21							320					450		470		530			630														2400
22							320					450		470		530			630														2400
20	230			290												530									830						1700	3580
31					300						400					530								800						1600			3630
28		250														530														1600			2380
18																								800							1700		2500
4									340						480	530	550	600															2500
37								330						470		530	550	600															2480
7																530						700						1250					2480
30																530							790				1100						2420
16	230						320									530														1350			2430
34					300		320									530													1300				2450
2					300											530							790		830								2450
19												450				530					690		790										2460
12		250							340	390		450		470				600															2500
26									340		400				480	530	550									900	1100						4280

Manual readout of the *UreAB/HaeIII* restriction grouped into highlighted band-types.
The numbers in the left column correspond to patient/strain numbers, the fragment-sizes of each individual patient are listed horizontally, with the sum of all fragments listed in the column on the far right.

Table 5

No	80	160	180	200	230	270	300	320	340	450	510	530	620	700	1220	sum
14			180	200				320	340					700		**1740**
21			180	200				320	340					700		**1740**
30			180	200				320	340					700		**1740**
16			180		230		300		340			530				**1580**
17			180		230		300		340			530				**1580**
32			180		230		300		340			530				**1580**
3			180						340		510	530				**1560**
8			180						340		510	530				**1560**
10			180						340		510	530				**1560**
19			180						340		510	530				**1560**
4			180						340			530		700		**1750**
6			180						340			530		700		**1750**
7			180						340			530		700		**1750**
9			180						340			530		700		**1750**
12			180						340			530		700		**1750**
15			180						340			530		700		**1750**
18			180						340			530		700		**1750**
20			180						340			530		700		**1750**
22			180						340			530		700		**1750**
23			180						340			530		700		**1750**
31			180						340			530		700		**1750**
35			180						340			530		700		**1750**
37			180						340			530		700		**1750**
26			180		230		300		340			530		700		**2280**
13			180		230		300		340					700		**1750**
29			180		230	270	300		340	450						**1770**
25			180	200			300		340			530				**1550**
28	80		180						340			530	620			**1750**
33			180						340		510	530		700		**2260**
2				200					340			530		700		**1770**
24		160	180						340			530				**1210**
34			180						340						1220	**1740**
36			180	200					340					700		**1420**

Manual readout of the UreCD/NdeII restriction grouped into highlighted band-types.
The numbers in the left column correspond to patient/strain numbers, the fragment-sizes of each individual patient are listed horizontally, with the sum of all fragments listed in the column on the far right.

Table 6

Patient/strain number	Conventional	GelComparII	Conventional	GelComparII
	Identical band patterns in *UreAB/HaeIII* digestion		**Identical band patterns in *UreCD/NdeII* digestion**	
2	19	Singular	Singular	22
3	Singular	Singular	8, 10, 19	8, 10
4	37	Singular	6, 7, 9, 12, 15, 18, 20, 22, 23, 31, 35, 37	7, 12, 18, 23, 31
6	36	Singular	4, 7, 9, 12, 15, 18, 20, 22, 23, 31, 35, 37	9
7	30	Singular	4, 6, 9, 12, 15, 18, 20, 22, 23, 31, 35, 37	4, 12, 18, 23, 31
8	13, 32	Singular	3, 10, 19	3, 10
9	14, 16, 17	14, 16	4, 6, 7, 12, 15, 18, 20, 22, 23, 31, 35, 37	6
10	Singular	Singular	3, 8, 19	3, 8
12	Singular	Singular	4, 6, 7, 9, 15, 18, 20, 22, 23, 31, 35, 37	4, 7, 18, 23, 31
13	8, 32	32	Singular	Singular
14	9, 16, 17	9, 16	21, 30	Singular
15	34	Singular	4, 6, 7, 9, 12, 18, 20, 22, 23, 31, 35, 37	Singular
16	9, 14	9, 14	17, 32	Singular
17	Singular	Singular	16, 32	Singular
18	Singular	Singular	4, 6, 7, 9, 12, 15, 20, 22, 23, 31, 35, 37	4, 7, 12, 23, 31
19	2	Singular	3, 8, 10	Singular
20	Singular	Singular	4, 6, 7, 9, 12, 15, 18, 22, 23, 31, 35, 37	37
21	22	Singular	14, 30	Singular
22	21	Singular	4, 6, 7, 9, 12, 15, 18, 20, 23, 31, 35, 37	2
23	Singular	Singular	4, 6, 7, 9, 12, 15, 18, 20, 22, 31, 35, 37	4, 7, 12, 18, 31
24	29, 35	Singular	Singular	Singular
25	33	Singular	Singular	Singular
26	Singular	Singular	Singular	Singular
28	Singular	Singular	Singular	Singular
29	24, 35	Singular	Singular	Singular
30	7	Singular	14, 21	Singular
31	Singular	Singular	4, 6, 7, 9, 12, 15, 18, 20, 22, 23, 35, 37	4, 7, 12, 18, 23
32	8, 13	13	16, 17	Singular
33	25	Singular	Singular	Singular
34	15	Singular	Singular	Singular
35	24, 29	Singular	4, 6, 7, 9, 12, 15, 18, 20, 22, 23, 31, 37	Singular
36	6	Singular	Singular	Singular
37	4	Singular	4, 6, 7, 9, 12, 15, 18, 20, 22, 23, 31, 35	20

Evaluation of band-patterns in both conventional manual and GelComparII analysis. Highlighted are the two only identical strains relying on manual analysis.

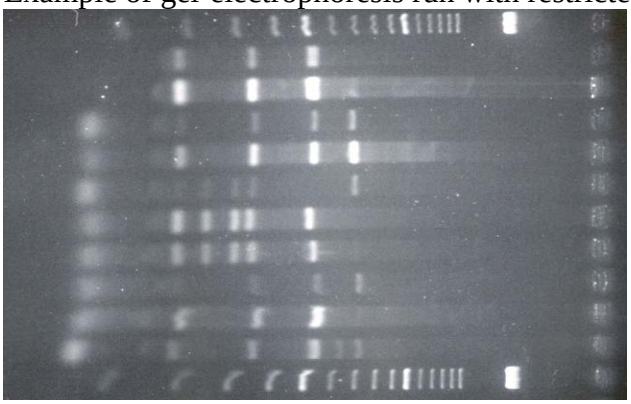
Table 7

	UreAB/HaeIII		***UreCD/NdeII***	
	manual	**GelComparII**	**manual**	**GelComparII**
Total amount of Band patterns	20	30	14	23
Band patterns with 2 or more strains	10	2	4	5
Singular band patterns	10	28	10	18

Number of band patterns after analysis.

Figure 1

Example of gel-electrophoresis run with restricted PCR-amplified segments of *H. pylori* DNA



The nucleic acids in the DNA wander from the wells through the gel towards the anode, the smaller the fragments the better they pass through the gel, enabling separation according to size

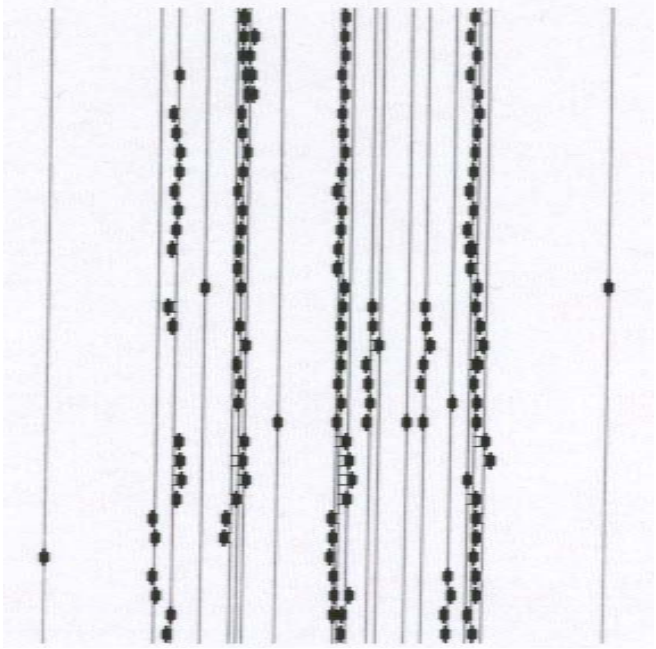
Figure 2

Electronically separated band

Using Applied Maths' program bands can be separated from the photos of electrophoresis gels and used as parts in a database. They are the basis for all information gained using the software.

Figure 3

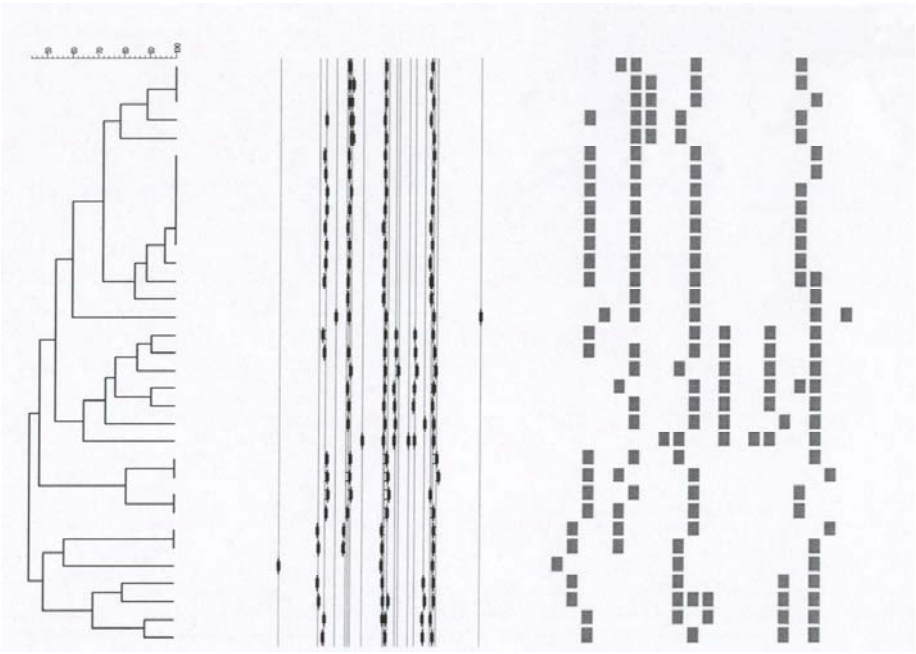
First step in evaluation with Applied Maths' GelComparII program



The bands highlighted by ethidium bromide are registered by the program and sorted according to base-pair-size. In this graph all patient samples are listed, ordered horizontally, displaying all band-sizes shown after restriction.

Figure 4

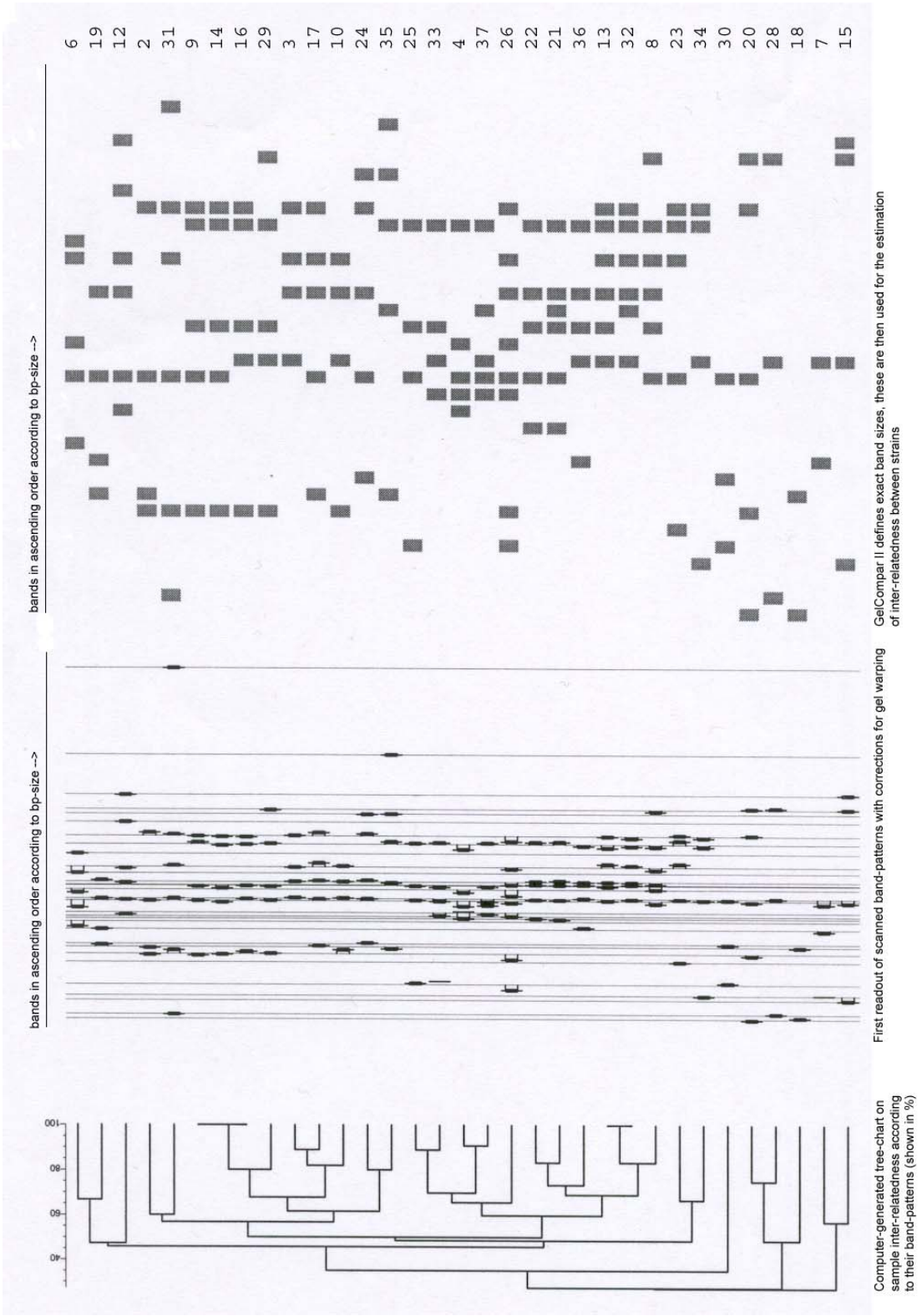
Result of analysis by GelComparII



After the last step of analysis, definition and correction of automatically assumed fragment-sizes, this is the resulting chart. From left to right three different graphs are shown, the middle one corresponding to **Figure 3**. On the right are shown the standardized boxes indicating the different fragment-sizes in each gel-electrophoresis analysed (strains ordered horizontally, size increasing from left to right). This graph is then used by GelComparII to create the graph on the far left, a tree showing the genetic similarity and assumed interrelatedness of all samples on which this restriction was performed.

Figure 5

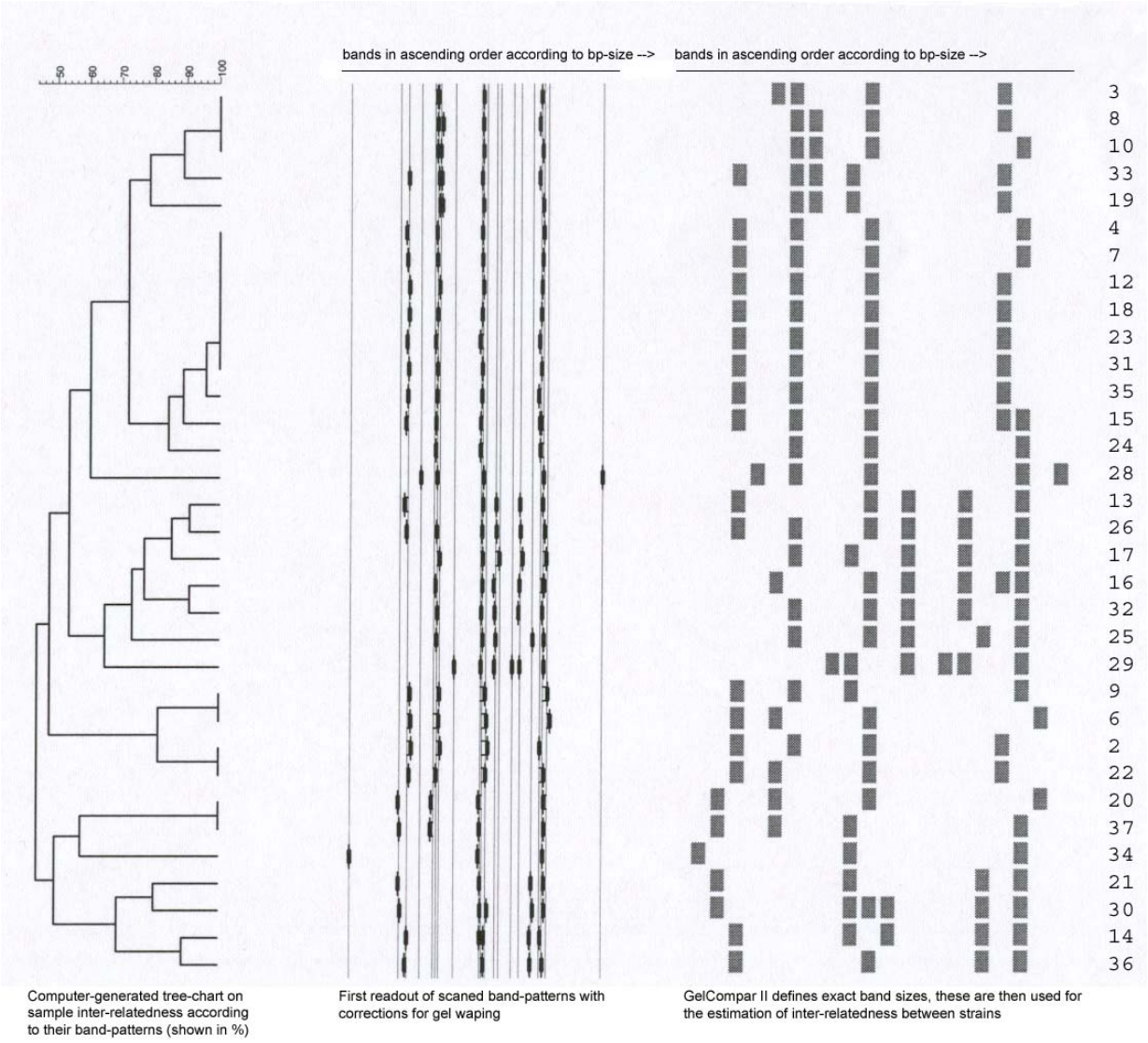
Full printout after analysis of the *UreAB*/*HaeIII* restriction band-patterns by GelComparII



Details are given in the figure itself; the chart represents a printout of all data available after analysis in GelComparII (Applied Maths, Belgium), the numbers correspond to patient samples.

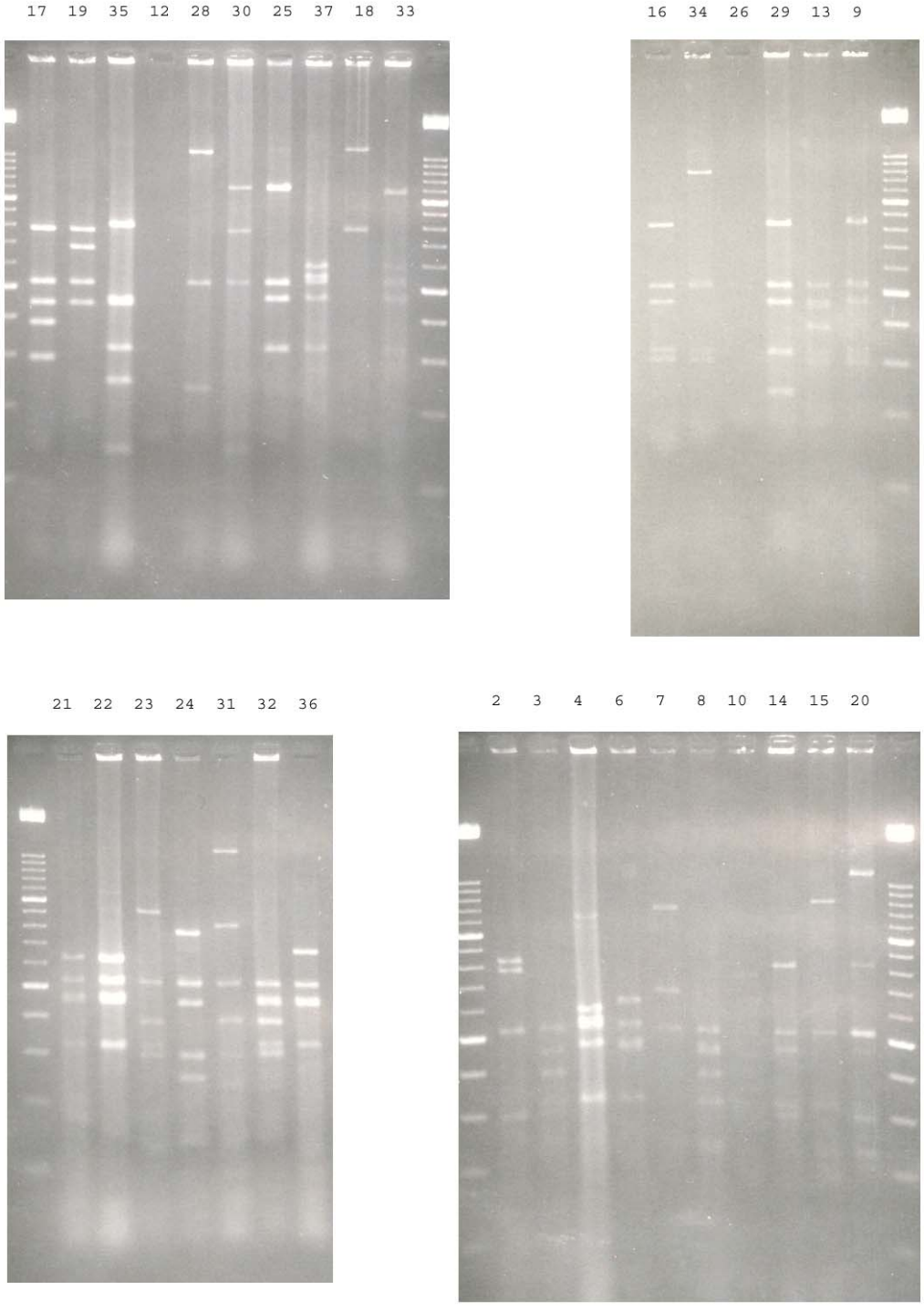
Figure 6

Full printout after analysis of the *UreCD*/*NdeII* restriction band-patterns by GelComparII



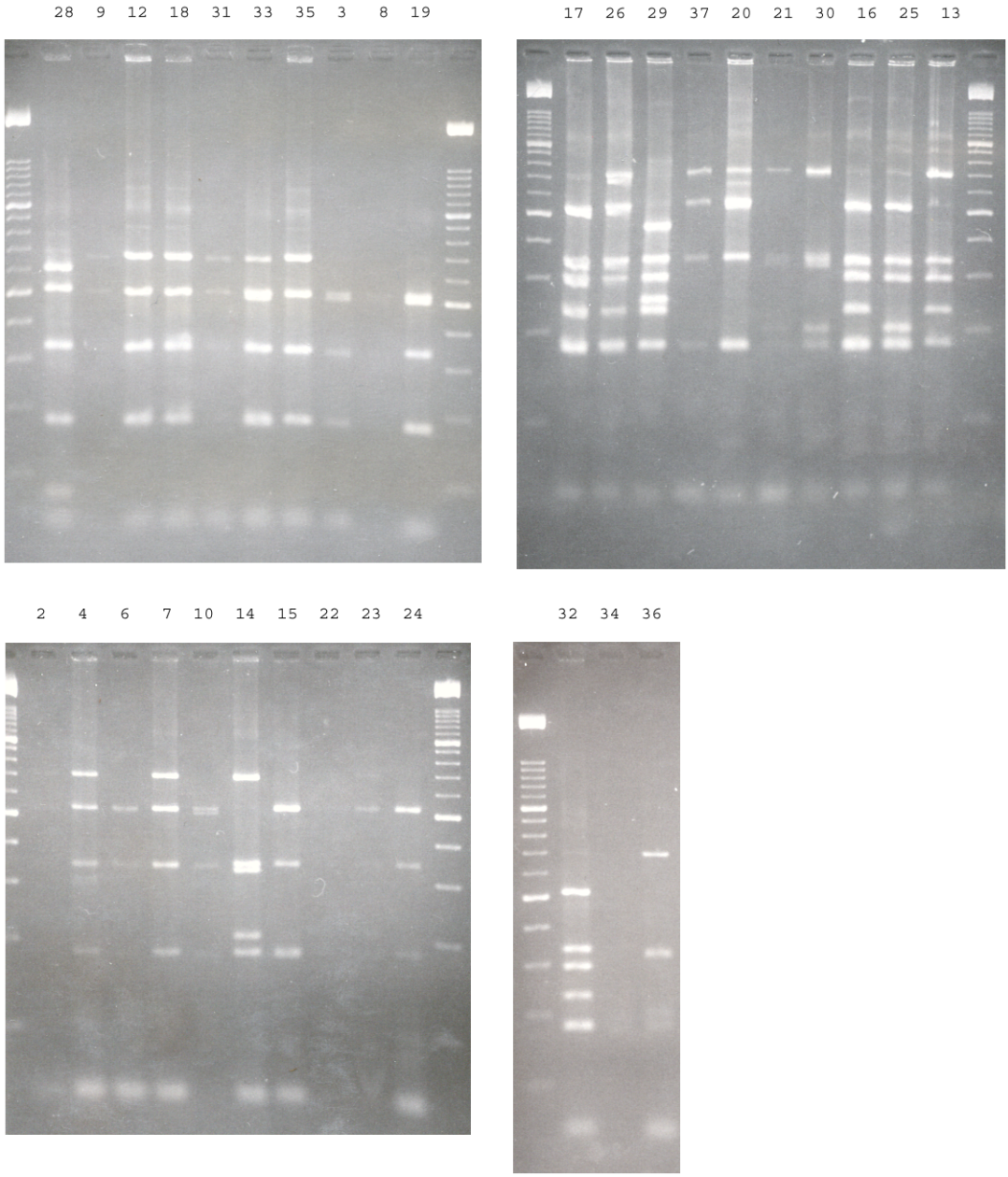
Details are given in the figure itself; the chart represents a printout of all data available after analysis in GelComparII (Applied Maths, Belgium), the numbers correspond to patient samples.

Figure 7



Scanned Polaroid pictures of a selection of band patterns after *UreAB/HaeIII* digestion samples were subjected to gel electrophoresis and ethidium bromide staining, numbers corresponding to patient samples.

Figure 8



Scanned Polaroid pictures of a selection of band patterns after *UreCD/NdeII* digestion samples were subjected to gel electrophoresis and ethidium bromide staining, numbers corresponding to patient samples.

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